VIRUS RESISTANT PAPAYA PLANTS DERIVED FROM TISSUES BOMBARDED WITH THE COAT PROTEIN GENE OF PAPAYA RINGSPOT VIRUS

Maureen M. M. Fitch[1,2]*, Richard M. Manshardt[1], Dennis Gonsalves[3], Jerry L. Slightom[4] and John C. Sanford[5]

[1]Department of Horticulture, University of Hawaii, Honolulu, Hawaii 96822. [2]Present address: U.S. Department of Agriculture, ARS, P.O. Box 1057, Aiea, Hawaii 96701. [3]Department of Plant Pathology, Cornell University, Geneva, New York 14456. [4]The Upjohn Company, Molecular Biology Unit 7242, Kalamazoo, Michigan 49007. [5]Department of Horticultural Sciences, Cornell University, Geneva, New York 14456. *Corresponding author.

Papaya ringspot virus (PRV) is a serious disease of papaya (*Carica papaya* L.) that has only been partially controlled by conventional methods. An alternative control method is coat protein-mediated protection (CPMP) through the transfer and expression of the PRV coat protein (*cp*) gene in papaya. We report an efficient gene transfer system utilizing microprojectile-mediated transformation of 2,4-D-treated immature zygotic embryos with a plasmid construction that contains the neomycin phosphotransferase II (NPTII) and β-glucuronidase (GUS) genes flanking a PRV *cp* gene expression cassette. Putative transgenic R_0 papaya plants, regenerated on kanamycin-containing medium, were assayed for GUS and PRV coat protein expression, for the presence of NPTII and PRV *cp* genes [with the polymerase chain reaction (PCR) and genomic blot hybridization analysis], and for PRV *cp* gene transcripts by Northern analysis. Four R_0 transgenic plant lines that contained the PRV *cp* gene showed varying degrees of resistance to PRV, and one line appeared to be completely resistant. These results represent the first demonstration that CPMP can be extended to a tree species such as papaya.

Papaya is one of the most widely grown fruit crops in the tropics and subtropics. The flavorful, melon-like fruit are rich in vitamins A and C and, when green, are the source of papain[1]. Papayas are produced commercially in plantations and on a smaller scale in dooryard gardens. However, papaya production is limited in many areas of the world due to the disease caused by papaya ringspot virus (PRV)[2]. PRV produces distinct ringspots on fruits, stunting of plants and leads to reduction in crop acreage (Fig. 1). The pathogen is a potyvirus, and control is difficult because PRV is normally transmitted by aphids in a nonpersistent manner[3]. Potyvirus constitute the largest and economically most important plant virus group[4].

There is little genetic resistance to PRV in papaya germplasm. Large collections of papaya lines and cultivars representing the world's major production areas have been screened, but resistant plants have not been found[5]. Varying degrees of tolerance have been observed, and one of the selections has been, or is being, used in breeding programs[6], but conventional breeding programs are expected to result in a compromise between useful resistance and acceptable fruit quality.

High levels of resistance to PRV are known to exist in several wild *Carica* species[5,7]. Interspecific hybrids between papaya and PRV resistant species have been produced with the aid of embryo rescue or ovule culture techniques[7-10], and in Hawaii, several F1 interspecific hybrids and a sesquidiploid produced by backcrossing to papaya were vigorous and showed excellent field resistance to PRV (R. Manshardt, unpublished data). However, these plants were quite sterile, and it seems that interspecific reproductive barriers will make the incorporation of resistance genes difficult.

PRV HA 5-1, a cross-protecting mild mutant strain of PRV that was selected following nitrous acid treatment of a severe strain from Hawaii[11], has been tested extensively in the field and is now used commercially in Taiwan[12,13] and Hawaii[14] to permit an economic return from papaya production. Cross protection, the deliberate infection of a crop with a mild viral strain to limit economic damage by more virulent strains, has several drawbacks, including a requirement for a large-scale inoculation program, a reduction in crop yield, and losses of cross-protected plants due to superinfection by virulent strains[15].

In order to overcome these problems, we investigated the potential of "pathogen-derived resistance"[16] via coat protein (*cp*) gene transformation, an approach first demonstrated by Powell Abel et al.[17] to delay the onset of severe symptoms of tobacco mosaic virus (TMV) in

FIGURE 1 PRV-infected papaya on the Island of Hawaii. (Photo courtesy of Wayne Nishijima, University of Hawaii, Hilo.)

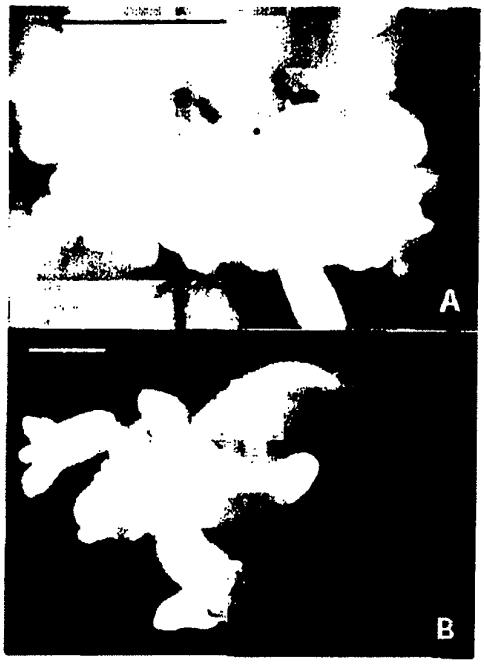


FIGURE 2 Embryogenic papaya tissues after particle bombardment. Frame (A) Histochemical GUS expression (blue dot) on the highly embryogenic apex of an immature zygotic embryo that had been treated with 2,4-D for 28 days prior to bombardment. The tissue was assayed four weeks after bombardment. Scale = 1.0 mm. Frame (B) Somatic embryos from the embryogenic apex of an immature zygotic embryo like that shown in Frame (A), growing selectively on kanamycin-containing medium. This is isolate S55-1 observed eight months post bombardment. Several selectively growing somatic embryos are shown adjacent to the brown cotyledon of original zygotic embryo. Scale = 1.0 mm.

transgenic tobacco. This coat protein-mediated protection (CPMP) against virus has since been found to be effective in protecting tobacco, tomato, or potato from infection by many different viruses (see Beachy et al.[18] for review) including PRV[19].

Ling et al.[19] demonstrated in tobacco that the expression of the PRV *cp* gene, isolated from the cross-protecting mild mutant strain PRV HA 5-1[20], afforded a broad spectrum of protection. The onset of viral symptoms was delayed in plants inoculated with three related potyviruses, tobacco etch (TEV), potato virus Y (PVY), and pepper mottle (PeMV). This construct provides a model system that allows direct comparison of the effectiveness of classical cross protection versus CPMP in controlling PRV in papaya. We recently developed papaya regeneration methods using embryogenic calluses and successfully transformed papaya via the biolistic method[21] with a vector containing NPTII and GUS genes and the *cp* gene of PRV HA 5-1. Transgenic papaya embryos and plants expressed the NPTII and GUS genes[22]. In this study, we show that a number of transgenic papaya lines contain the *cp* gene of PRV HA 5-1 and that these plants show varying degrees of resistance to inoculation with the severe Hawaiian strain PRV HA[23]. One line is completely resistant. These results improve the prospects for papaya cultivation in areas now abandoned due to PRV infestation.

TABLE 1 Characterization of transgenic papaya plants for the presence of GUS expression and PCR-amplification of Nos-NPTII and PRV *cp* gene fragments. Plants from embryogenic calluses and somatic embryos from hypocotyls (EC) and 2,4-D-treated zygotic embryos (ZE), that yielded putative transgenic tissues, were assayed.

Tissue type	Total number of positive plants/total number assayed		
	GUS expression	NPTII (PCR)	PRV *cp* (PCR)
EC	3/5 (60%)	3/4 (75%)	3/5 (60%)
ZE	9/25 (36%)	18/18 (100%)	7/19 (37%)
Total	12/30 (40%)	21/22 (95%)	10/24 (42%)

RESULTS

Papaya target tissues and selection of transgenic papaya by growth on kanamycin. Three types of papaya tissues, including papaya hypocotyl sections (H), embryogenic calluses (EC), and 2,4-dichlorophenoxyacetic acid-(2,4-D) treated zygotic embryos (ZE) were tested to determine which type would regenerate the most transgenic plants. A total of 70 petri dishes of papaya tissues from commercial cultivars "Sunset" (S) or "Kapoho" (K) were bombarded with microprojectiles coated with pGA482GG/cpPRV-4 DNA[22], and putative transformed papaya embryos (Fig. 2) were isolated on selection medium containing 150 mg/l kanamycin over a period of four to 23 months. Twenty-five of the plates yielded at least one transformed cell line, 55 different cell lines grew selectively on kanamycin-containing medium, and 30 plant lines were regenerated.

Freshly explanted papaya hypocotyl sections were not suitable tissue targets for microprojectile-mediated transformation; only one GUS$^+$ embryogenic callus was observed, but it ceased growth. None of the remaining hypocotyl sections produced a kanamycin resistant callus during a year of culture. Embryogenic callus cultures, the simplest tissues to prepare for bombardment, yielded several selectively growing embryo clusters. Seventeen percent of the cultures subjected to bombardment gave rise to a total of 20 kanamycin resistant embryo clusters over a two-year culture period. The efficiency on a fresh weight (FW) basis was 1.14 selectively growing callus lines/g FW of bombarded tissues. However, the regeneration of plants from these potentially transformed calluses was difficult because many of the embryo lines developed into abnormal structures rather than shoots. Only five lines regenerated plants, three of which produced abnormal shoots with broom-shaped leaves that resembled damage due to virus- or herbicide-induced effects. The two other plant lines, K19-1 and S33-2, appeared normal.

Immature zygotic embryo cultures, the most difficult to prepare, yielded the largest number of transgenic embryo lines that subsequently regenerated into plants (Table 1). Three fourths of the 24 petri dishes of bombarded zygotic embryos produced at least one transgenic embryo line (Fig. 2). With about 100 zygotic embryos per bombarded dish, the transformation efficiency was about 1.42% of the zygotic embryos. Of the 34 putative transgenic embryo lines, 74% regenerated normal-looking plants, while the other 26% was lost due to cessation of growth on kanamycin-containing medium.

The regeneration of papaya plants from the putatively transformed zygotic embryos was a complex process. Some cell lines grew vigorously and regenerated in the presence of 150 mg/l kanamycin (Fig. 2B), while the growth of others was inhibited. The latter were only capable of regenerating plants after their removal from media containing kanamycin. Of the three different tissue types tested, transgenic papaya plants were established from only the embryogenic calluses and the 2,4-D-treated zygotic embryos.

Identification of transgenic R_0 papaya plants: GUS expression, PCR and genomic and RNA blot analyses. About one third (9 out of 25, Table 1) of the regenerated plants from the zygotic embryos were GUS$^+$ in histo-

chemical assays of young leaves. Leaves of regenerated plants were screened for GUS because embryos sometimes produced "false positive", light blue, irregular spot patterns when exposed for more than 12 hours to the histochemical substrate. Since untransformed leaves never turned blue, histochemical data from leaves were the most reliable. Figure 3A shows the strong, uniform GUS expression in a leaf derived from plant S55-1. However, GUS expression often varied between individual plants and within the same plant. For example, cut leaves from plants K44-1, S55-1, and S60-4 consistently stained dark blue at all injured surfaces. On the other hand, plant K41-1 stained intensely blue as selectively growing somatic embryos and calluses (see Fig. 2F in Fitch et al.[23]), but fully expanded leaves were GUS$^-$ (data not shown). Only the youngest leaves, about 1/3 fully expanded, turned pale blue after 3 to 4 hours in the histochemical assay. Similar results were found among the other putatively transformed papaya plants, which suggested that the GUS gene was being expressed at different levels in these plants. Leaves of some plants stained most intensely in the vascular tissues and petioles (plant S59-1 and K19-1, data not shown), while others stained in circular spot or wedge-shaped patterns on the lamina of young leaves (S60-3, Fig. 3B) but not on the older leaves.

DNAs isolated from all of the GUS$^+$ and several of the GUS$^-$ papaya plants were tested for the presence of the Nos-NPTII gene of pGA482[24] using the PCR amplification procedure described by Chee et al.[25]. A 1.0 kb DNA fragment was amplified in all putative transgenic plants (data not shown). Genomic Southern blot analysis of DNAs isolated from several R_0 papaya plants showed the characteristic 2.0 kb BamHI/HindIII fragment[24] containing the Nos-NPTII gene in most plants (Fig. 4). In addition, evidence for multiple or rearranged copies of the Nos-NPTII gene is shown by the varying intensities of the 2.0 kb bands and by additional bands, both larger and smaller than 2.0 kb.

Due to the random nature of the DNA integration event that follows microprojectile bombardment[26,27], papaya plants found to contain either the Nos-NPTII or GUS genes or both did not necessarily contain the PRV *cp* gene, even though the PRV *cp* gene was located between the Nos-NPTII and GUS genes in the plasmid vector pGA482GG/cpPRV-4[15,22]. Thus, the presence or absence of the PRV *cp* gene expression cassette in putatively transformed papaya plants was established using both PCR and genomic Southern blot analyses. Genomic DNAs isolated from putatively transformed papaya plants were subjected to PCR, using two oligonucleotide primers that amplify a PRV *cp* gene DNA fragment of about 1.0 kb in length. Ten out of 12 GUS$^+$ plants were PRV *cp* gene$^+$ (PCR), K19-1, K27-1, K29-1, K39-1, K41-1, S49-2, S55-1, S59-1, S60-1, and S60-3 (Table 1). The remaining two GUS$^+$ plants, K44-1 and S60-4, were PRV *cp* gene$^-$ (PCR). PCR analysis (Fig. 5, lanes 6 to 8) showed the absence of PRV *cp* gene amplification in GUS$^+$ plant K44-1 as well as in two GUS$^-$ plants, S54-1 and S62-5.

Additional support for the presence of the PRV *cp* gene in the genome of transgenic plants was established by genomic blot analysis of BamHI/HindIII digests. The results of hybridization against a PRV *cp* gene probe are shown in Figure 6. DNAs isolated from each of the PRV *cp* gene$^+$ plants, S55-1, S59-1, and K39-1, showed the presence of the 1.7 kb HindIII fragment that contains the PRV *cp* gene expression cassette[19,20,22], but DNAs from plants S62-1 and S62-2, determined by PCR to be PRV *cp* gene$^-$, did not hybridize with the PRV *cp* gene probe.

Transcripts from two PRV *cp* gene$^+$ plants, S55-1 and S60-3, were detected in an analysis of total RNA (Fig. 7).

FIGURE 3 Papaya leaves, sliced from the midrib to the margins and bruised with forceps to expose cells to the X-gluc substrate. Frame (A) Strong, uniform GUS expression in slices and bruises on S55-1. Frame (B) Unique pattern of GUS$^+$ spots on S60-3. Spots are not due to injury since slices and bruises on this leaf did not show strong GUS expression of S55-1 in Frame (A).

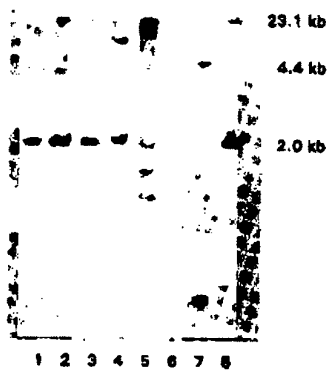


FIGURE 4 Genomic Southern blot analysis for the presence of the Nos-NPTII gene in putative transgenic papaya plants. Hybridization of BamHI and HindIII digests of papaya DNAs and pGA482GG with a probe for the NPTII gene. Lane 1: S55-1 (GUS$^+$), lane 2: S59-1 (GUS$^+$), lane 3: S59-2 (GUS$^-$), lane 4: S63-1 (GUS$^-$), lane 5: S60-4 (GUS$^+$), lane 6: S33-2 (GUS$^-$), lane 7: S64-1 (GUS$^-$), and lane 8: pGA482GG. The NPTII gene probe hybridized to a characteristic band at 2.0 kb in six out of seven plant samples and in the digested plasmid DNA. Hybridizing bands larger than 2.0 kb may have resulted from incomplete digestion of the DNAs or to rearranged genes. DNAs in samples S60-4 and S64-1 apparently underwent considerable rearrangement.

The predicted transcript at 1.35 kb was observed in both plants, but S55-1 contained, in addition, larger transcripts at 2.4 and 4.4 kb.

ELISA assay of PRV *cp* gene expression. Initially, to detect PRV CP, ELISA tests using polyclonal antibodies

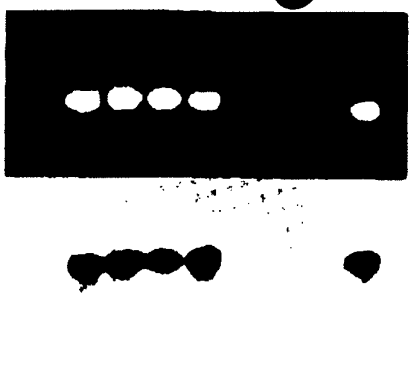


FIGURE 5 PCR detection of the PRV *cp* gene in transgenic papaya plants. Frame (A) An ethidium bromide-stained gel showing the 1.0-kb PRV *cp* gene fragment. Frame (B) Gel from Frame (A) blotted and hybridized with the PRV *cp* gene probe. Lane 1: untransformed papaya, lanes 2 to 9: transgenic papayas, 2: S55-1 (GUS+), 3: K19-1 (GUS+), 4 and 5: K29-1 (GUS+), 6: K44-1 (GUS+), 7: S54-1 (GUS−), 8: S62-5 (GUS−), and 9: S60-3 (GUS+). The 1.0-kb PRV *cp* gene fragment was amplified in three out of four GUS+ plant lines, while no amplification occurred in the untransformed control and in GUS-lines.

FIGURE 6 Genomic Southern blot hybridization of BamHI and HindIII digests of papaya DNAs with a probe for the PRV *cp* gene. Both restriction enzymes were used to digest the DNAs because filters were probed twice, once for the presence of the Nos-NPTII gene and secondly for the PRV *cp* gene. The characteristic 2.0 kb fragment for Nos-NPTII is flanked by BamHI and HindIII[28], while the 1.7 kb PRV *cp* gene fragment is flanked by HindIII[19]. Lane 1: untransformed papaya, lane 2: S62-1 (GUS−), lane 3: S62-2 (GUS−), lanes 4 and 5: S55-1 (GUS+), lane 6: S59-1 (GUS+), lanes 7 and 8: K39-1 (GUS+). The PRV *cp* gene probe hybridized to the predicted 1.7 kb HindIII fragment in S55-1, SS59-1 and K39-1 that previously were found to be GUS+ and PRV *cp* gene+ with PCR.

TABLE 2 Reaction of subcloned transgenic R_0 papaya plant lines to inoculation with PRV HA.

R_0 Line	GUS	PCR *cp*	HT (cm)	No. Infected/ No. Inoculated	Percent Infected	Delay in symptoms[a]
S55-1	+	+	5-11	0/11	0	—
S55-1	+	+	25-28	0/2	0	—
S60-3	+	+	7-12	3/3	100	6-15
S60-3	+	+	20-48	0/9	0	—
K19-1	+	+	6-9	3/4	75	3-17
K19-1	+	+	14-25	1/8	13	0
K39-1	+	+	8-12	10/10	100	0-7
K39-1	+	+	11-46	4/5	80	0-15
S33-2	−	−	13-14	3/3	100	0
S54-1	−	−	14-50	9/9	100	0
S62-1	−	−	4-13	9/9	100	0
S62-5	−	−	14-37	5/5	100	0
K44-1	+	−	11-50	9/9	100	0
Control	−	−	8-28	35/35	100	0

[a]The delay in symptoms is estimated by using the time of symptom expression in controls as "0" days. HT = plant height when inoculated.

for coating and in the conjugate, were performed on *in vitro*-grown plants, K29-1, K39-1, and S55-1, that contained the PRV *cp* gene. These tests were inconclusive because the transgenic plants gave absorption readings that averaged only 1.0–1.6-fold above the relatively high background readings of healthy plants (A_{405} = 0.215). However, subsequent tests with vigorously growing S60-3 and S55-1 plants at the flowering stage gave positive results with ELISA tests using monoclonal antibody conjugates that eliminated background reactions. S55-1 gave an average absorption reading of 0.238, S60-3 gave a reading of 0.252, while healthy papaya had a reading of 0.001. These results clearly showed that the transgenic plants produced detectable levels of coat protein.

Protection of R_0 papaya plants against mechanical PRV infection. Nine micropropagated R_0 transgenic papaya plants were selected for testing PRV susceptibility under greenhouse conditions using mechanical inoculation of PRV (Table 2). Between three and 15 micropropagated plants derived from each of the nine R_0 plants were inoculated. Four of the plant lines contained the PRV *cp* gene expression cassette (K19-1, K39-1, S55-1, and S60-3), while the remaining five lines did not (K44-1, S33-2, S54-1, S62-1, and S62-2). These plants, along with 35 untransformed control plants, were mechanically inoculated with PRV HA, the parent strain of the mild mutant that has been used for classical cross protection[11,12,20]. Papaya plants infected with PRV HA show chlorosis and leaf distortion, water-soaked streaks on the stem, and stunted growth.

The results listed in Table 2 indicate that the PRV *cp* gene+ papaya lines show varying levels of virus protection, as judged by the number of inoculated plants that became infected. The levels of protection observed included no protective effect in line K39-1, an intermediate level of resistance, indicated by a delay in the onset of symptom development in lines K19-1 and S60-3, and apparently complete resistance in line S55-1 (Table 2). Inoculated plants of line S55-1 did not show signs of infection on the mechanically inoculated leaves nor on leaves that subsequently developed during maturation of the plant (Fig. 8). Tests to recover PRV from the inoculated S55-1 plants by means of transferring leaf extracts to a local lesion host (*Chenopodium quinoa*) were negative, indicating complete resistance afforded by the apparent inhibition of PRV replication. The micropropagated plants derived from S55-1 remained symptomless for the duration of the experiment which lasted up to six months (Fig. 8D). Several of the symptomless plants were retained for seed production and have remained symptomless for more than nine months.

The PRV *cp* gene+ lines K19-1 and S60-3 were characterized by intermediate levels of protection since 25 to 33% of the total number of inoculated plants became infected (Table 2). Interestingly, the plants that became infected showed delays in the onset of symptom expression ranging from three to 17 days. We also observed that the plants that became infected were generally inoculated at a smaller stage of growth (Table 2). The lack of infection of the larger K19-1 and S60-3 plants was not strictly due to size, since untransformed control plants of comparable size invariably became infected. As with papaya line S55-1, tests to recover PRV from symptomless K19-1 and S60-3 plants were negative. Although papaya line K39-1 proved to be completely susceptible to infection by PRV HA, individual plants showed delays in the onset of symptom development. All of the transformed papaya plant lines that tested negative for the presence of the PRV *cp* gene were susceptible to PRV HA infection, and their symptoms appeared at about the same time as

did the symptoms in the inoculated untransformed control plants.

DISCUSSION

The number of transgenic papaya plants regenerated was variable between tissues derived from zygotic embryos and hypocotyls. The most efficient recovery of plants followed microprojectile bombardment of 2,4-D-treated immature zygotic embryos, while freshly explanted hypocotyl sections did not yield any transgenic plants.

Neither GUS expression nor PRV *cp* gene assays were completely reliable predictors of virus resistant plants, although the assays were useful in initial screening of transgenic plants. Even transcript analysis and levels of PRV CP production were not predictive. We found that the only reliable indicator of CPMP was the functional test, *i.e.*, the infection of the transgenic papaya plants with a virulent strain of PRV. The decrease in GUS expression in older papaya leaves and the variations in the level of PRV protection are not understood at this time. A resolution of these questions can only be achieved by determining the transformed states (gene copies, arrangements, etc.) in each plant, and by using breeding techniques to obtain homozygous plants that contain a known arrangement of transferred genes.

Despite the small number of transgenic plants recovered, functional analysis of only nine plant lines resulted in the identification of S55-1 which is apparently completely resistant to PRV HA, the virulent Hawaiian strain from which the cross-protecting mutant was isolated. Since the PRV *cp* gene was obtained from the virus strain identical to that used for cross protection, the initial results with CPMP can be compared with classical cross protection. We assume that our plant lines are not chimeric; therefore, unlike the cross-protecting virus, the protection afforded by the presence of the *cp* gene is systemic. Unless a developmental factor governs the protective element of the *cp* gene, we expect no breakdown in CPMP. Cross protection with live virus, a practice that becomes questionable in cool weather when even mild strain symptoms can be pronounced, is circumvented with CPMP. A major benefit of CPMP is heritability of protection, eliminating manual inoculation of each new crop. Finally, it is possible that even greater protection

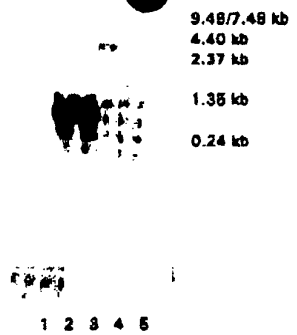


FIGURE 7 Northern blot of PRV *cp* gene+ transgenic papayas. Lane 1: untransformed papaya, lanes 2 and 3: S60-3, and lanes 4 and 5: S55-1. The bands at 1.35 kb correspond to the predicted transcript size.

can be afforded by the homozygous gene condition in R_1 or R_2 plants.

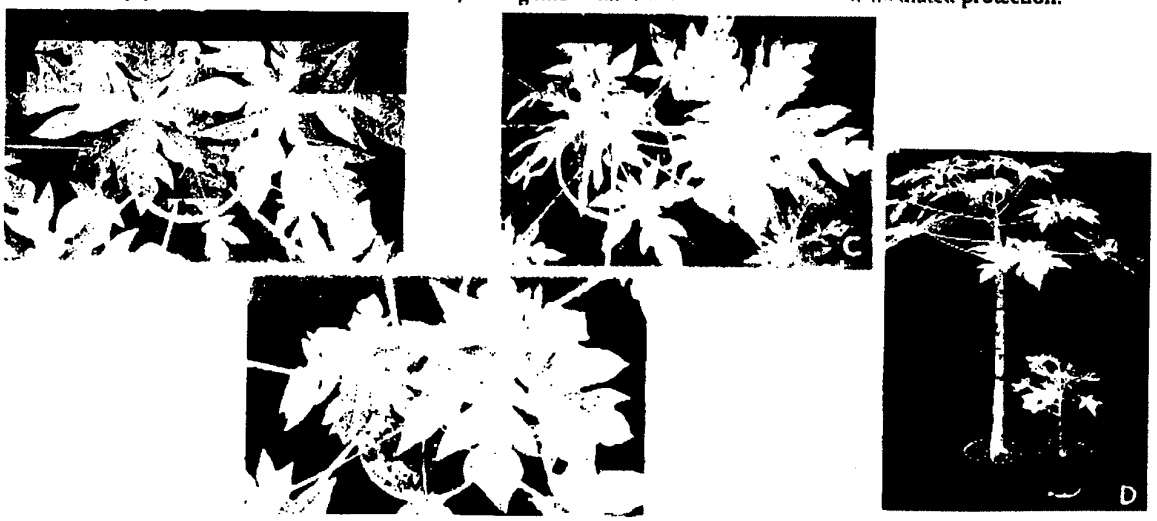
The virus resistant line S55-1 is female; thus it has been outcrossed with hermaphroditic papayas such as S60-3, that showed an intermediate level of resistance (Table 2), and with untransformed controls. A 1:1 segregation for sex expression, female:hermaphrodite, is expected in the progeny from these crosses[30]. Homozygosity will fix the PRV *cp* gene in papaya lines after R_1 hermaphrodites containing the PRV *cp* gene are selfed. Preliminary data indicate that seedlings from outcrossed S55-1 show the expected 1:1 segregation of a single insertion of the three transgenes, GUS, NPTII, and PRV *cp* (S. Liu, unpublished data). It is possible that some of the hermaphrodite R_1 progeny will be suitable for commercial use even in the hemizygous state, if they prove to be totally virus resistant.

Since we do not know how well or how long CPMP will remain effective in our PRV resistant plant lines, the R_0 virus resistant line has been installed in a field test in Hawaii to determine whether the protection observed in the greenhouse tests can withstand prolonged exposure (two to three years) to PRV under the continuous challenge of virus inoculation by the natural aphid vector.

EXPERIMENTAL PROTOCOL

Materials. Restriction endonucleases BamHI, BglII, EcoRI, HindIII, and NcoI were purchased from Gibco/BRL, Grand

FIGURE 8 Virus screening of transgenic papaya plants. Frame (A) Virus resistant S55-1, uninoculated, photographed three months after initiation of the experiment. Frame (B) Virus resistant S55-1, inoculated with PRV HA, a virulent Hawaiian strain of PRV and photographed three months after inoculation. This transgenic plant appears to be unaffected by the virus. Frame (C) Untransformed control "Sunrise", sibling line of "Sunset", inoculated with PRV HA and photographed three months after inoculation. Mottling and "shoestring" leaf development are typical severe virus symptoms. Frame (D) Papaya plants inoculated with PRV HA and photographed after six months. Left, virus resistant S55-1; right, untransformed control. The difference in plant height illustrates the resistance afforded [illegible] mediated protection.



land, NY. 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-gluc) was obtained from Jersey Lab Supply, NJ. Taq polymerase and PCR kits were obtained from Perkin-Elmer-Cetus Corporation. Random priming kits for ^{32}P-labeling and digoxigenin-labeling were obtained from Boehringer Mannheim. (α-^{32}P)dCTP was obtained from New England Nuclear Biolabs. Oligonucleotide synthesis was done using an Applied Biosystems Instrument Model 380A.

Plant materials and culture conditions. Cultures of freshly explanted hypocotyl sections, embryogenic calluses and somatic embryos, and 2,4-D-treated 90- to 105-day-old zygotic embryos were prepared for particle bombardment as described[28]. Immature zygotic embryos of "Kapoho" and "Sunset" were induced to embryogenesis on half-strength Murashige and Skoog[51] medium containing 10 mg/l 2,4-D[52]. Embryogenesis was induced in hypocotyl sections of "Kapoho" on the same medium[23].

Plasmid constructs and gene delivery. The construction of the binary cosmid pGA482GG/cpPRV-4 has been described[19,28,29]. Transfer of the construction with the Biolistics device has been described[22].

Recovery of transgenic embryos and plants. Transgenic somatic embryos were selected on induction medium containing kanamycin and 2,4-D as described[23]. Despite monthly transfers to fresh selection medium devoid of phytohormones, the sectors continued to undergo repetitive cycles of embryogenesis. Somatic embryos were germinated on MS medium containing 150 mg/l kanamycin. MS medium consisted of MS salts, 100 mg/l myo-inositol, 0.4 mg/l thiamine•HCl, 3% sucrose, and 0.5% Sigma A1296 agar, pH 5.8. Shoots from germinated embryos were micropropagated for rapid growth in liquid MPII medium[54]. Shoot tip cuttings with 1.0-cm long stems were rooted in MS agar medium containing 1.0 mg/l indolebutyric acid (IBA) and transferred to jars containing a 50/50 (v/v) mixture of vermiculite and liquid MS medium. Plants were acclimated to greenhouse conditions.

GUS histochemical assay. Leaves were sliced and incubated overnight at 37°C in filter-sterilized 0.5 mM 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-gluc) in 200 mM sodium phosphate buffer, pH 7.0[55]. Leaf tissues were cleared of chlorophyll after soaking in 95% ethanol to enhance visualization of the blue precipitate. Isolates that initially tested negative for GUS were re-tested at least five times before they were scored negative.

DNA extraction. DNA was extracted using "CTAB" methods[56,57]. Between 50 and 700 mg dry weight of tissues per sample were extracted. Each sample, containing up to 500 μg of DNA, was treated with 2000 units of RNase (DNase-free, Boehringer Mannheim) for 2 h at 37°C prior to quantification and further analysis.

Polymerase chain reaction. Genomic DNA was subjected to amplification by the polymerase chain reaction (PCR)[58]. One set of primers was designed to amplify a 1052 bp fragment of a chimeric gene for neomycin phosphotransferase II (NPTII). The fragment extends from the Nos promoter at the 5' end of the gene to 150 bp beyond its 3' termination sequence[59]. Another set of primers was designed to amplify a 992 bp PRV *cp* gene fragment[19,28,29]. Standard PCR conditions, as recommended by Perkin-Elmer-Cetus, were followed. PCR products were size-separated on 0.8% agarose gels.

Southern hybridization. Probes were prepared by large scale plasmid isolation[59]. Cesium chloride ethidium bromide centrifugation was used to isolate plasmid pKS4 containing the NPTII gene that was derived from *E. coli* transposon Tn5 (ref. 40). Plasmid DNA was digested with NcoI and BglII to release a 600 bp NPTII gene fragment[28]. The fragment was gel-purified on 1% agarose, electroeluted[59], and concentrated with an Elutip column (Schleicher and Schuell, Keene, NH) as recommended by the manufacturer. The PRV *cp* gene probe was prepared from pPRV117[29] by digesting it with EcoRI which released a 500 bp fragment from the 3' end of the gene. The fragment was electroeluted and concentrated as described. Isolated fragments of plasmid DNA were labeled with (α-^{32}P)dCTP or digoxigenin by random priming[41] according to the manufacturer's instructions (Boehringer Mannheim). Southern blots were prepared from agarose gel separations of PCR products or digested genomic DNAs[59]. Genomic DNAs from putative transgenic leaves were digested with six-fold excess of HindIII and BamHI, size-fractionated on 0.8 agarose gels, blotted onto nitrocellulose ("Duralose", Stratagene) for ^{32}P- or onto nylon (Boehringer Mannheim) for digoxigenin-labeled probes, and hybridized[59]. Scintillation counts of the incorporated radioactivity were about 5×10^6 *cpm*/100 cm^2 filter. Filters were hybridized for at least 48 h at 65°C with ^{32}P-labeled probes. Digoxigenin-labeled probes were hybridized for 24 to 48 h at 42°C in formamide hybridization solution, washed, and processed for chemiluminescence as recommended by Boehringer Mannheim. Filters hybridized against ^{32}P-labeled probes were exposed for two to four days to Kodak OMAT X-ray film; digoxigenin-probed filters were exposed to X-ray film for 15 to 60 min.

Northern hybridization. Total RNA was isolated from leaves of untransformed and transgenic papaya plants by the method of Napoli et al.[42] and separated on a 1.2% agarose gel (25 μg/lane) using formaldehyde gel electrophoresis[43]. The separated RNAs were blotted onto a GeneScreen Plus membrane and probed with the *cp* gene of PRV following the manufacturer's manual (Du Pont Co.). The probe was prepared by random primer labeling as described by Feinburg and Vogelstein[41]. The filter was exposed for 1.5 h.

ELISA assays for presence of PRV CP. Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) technique[44], employing a polyclonal antibody and monoclonal antibody conjugate, was used to assay for PRV CP in putative transgenic leaves and in infected controls[23].

Infection of transgenic plants with PRV. Micropropagated papaya plants derived from R_0 plants known to contain the PRV *cp* gene sequence were grown in the greenhouse until they bore four or five leaves. The plants were dusted with 400 mesh carborundum on the four youngest expanded leaves. The leaves were rubbed with 50 μl of a 1/50 dilution of PRV HA-infected *Cucumis metuliferous* leaf extract in 0.01 M potassium phosphate buffer, pH 7.0[11]. The inoculum was prepared 21 days after *Cucumis* infection. The plants were visually monitored daily for 21 days at which time the results were summarized (Table 2). Plants that did not show symptoms were retained for continued observation. The sap was extracted from leaves of symptomless plants and applied to the virus indicator plant *Chenopodium quinoa* to screen for the presence of virus. Plants were tested by ELISA to detect antigens of PRV.

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References

1. Purseglove, J. W. 1968. Caricaceae, p. 45–51. *In:* Tropical Crops, Vol. 1: Dicotyledons. Longman Group, Ltd. Essex, UK.
2. Purcifull, D. E., Edwardson, J. R., Hiebert, E. and Gonsalves, D. 1984. Papaya ringspot virus. CMI/AAB Descriptions of Plant Viruses, no. 84, revised.
3. Conover, R. A. 1964. Distortion ringspot, a severe virus disease of papaya in Florida. Proc. Fl. State Hort. Soc. 77:440–444.
4. Hollings, M. and Brunt, A. A. 1981. Potyviruses, p. 731–807. *In:* Handbook of Plant Virus Infection and Comparative Diagnosis. E. Kurstak (Ed.). Elsevier/North. Amsterdam, The Netherlands.
5. Conover, R. A. 1976. A program for development of papayas tolerant to the distortion ringspot virus. Proc. Fl. State Hort. Soc. 89:229–231.
6. Conover, R. A., Litz, R. E. and Malo, S. E. 1986. "Cariflora"—a papaya ringspot virus tolerant papaya for South Florida and the Caribbean. HortScience 21:1072.
7. Horovitz, S. and Jimenez, H. 1967. Cruzamientos interspecificos e intergenericos en caricaceas y sus implicaciones fitotecnicas. Agron. Trop. 17:525–545.
8. Manshardt, R. M. and Wenslaff, T. F. 1989. Zygotic polyembryony in interspecific hybrids of *Carica papaya* and *C. cauliflora*. J. Amer. Soc. Hort. Sci. 114:684–689.
9. Manshardt, R. M. and Wenslaff, T. F. 1989. Interspecific hybridization of papaya with other *Carica* species. J. Amer. Soc. Hort. Sci. 114:689–694.
10. Chen, M. H., Chen, C. C., Wang, D. N. and Chen, F. C. 1991. Somatic embryogenesis and plant regeneration from immature embryos of *Carica papaya* x *Carica cauliflora* cultured *in vitro*. Can. J. Bot. 69:1913–1918.
11. Yeh, S.-D. and Gonsalves, D. 1984. Evaluation of induced mutants of papaya ringspot virus for control by cross protection. Phytopathology 74:1086–1091.
12. Wang, H.-L., Yeh, S.-D., Chiu, R.-J. and Gonsalves, D. 1987. Effectiveness of cross-protection by mild mutants of papaya ringspot virus for control of ringspot disease of papaya in Taiwan. Plant Disease 71:491–497.

19. Yeh, S.-D., Gonsalves, D., Wang, H.-L., Namba, R. and Chiu, R.-J. 1988. Control of papaya ringspot virus by cross protection. Plant Disease 72:375–380.
14. Mau, R. F. L., Gonsalves, D. and Bautista, R. 1990. Use of cross protection to control papaya ringspot virus at Waianae. p. 77–84. *In:* Proceedings, 25th Annual Hawaii Papaya Industry Association Conference, 1989. C. L. Chia (Ed.). College of Tropical Agriculture and Human Resources, University of Hawaii, Honolulu, Hawaii.
15. Gonsalves, D. and Garnsey, S. M. 1989. Cross-protection techniques for control of plant virus diseases in the tropics. Plant Disease 73:592–597.
16. Sanford, J. C. and Johnston, S. A. 1985. The concept of parasite-derived resistance—deriving resistance genes from the parasite's own genome. J. Theor. Biol. 113:395–405.
17. Powell Abel, P., Nelson, R. S., De, B., Hoffman, N., Rogers, S. G., Fraley, R. T. and Beachy R. N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738–743.
18. Beachy, R. N., Loesch-Fries, S. and Tumer, N. E. 1990. Coat protein-mediated resistance against virus infection. Annu. Rev. Phytopath. 28:452–474.
19. Ling, K., Namba, S., Gonsalves, C., Slightom, J. L. and Gonsalves, D. 1991. Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. Bio/Technology 9:752–758.
20. Quemada, H., L'Hostis, B., Gonsalves, D., Reardon, I. M., Heinrickson, R., Hiebert, E. L., Sieu, L. C. and Slightom, J. L. 1990. The nucleotide sequences of the 3' terminal regions of papaya ringspot virus strains W and P. J. Gen. Virol. 70:203–210.
21. Sanford, J. C. 1990. Biolistic plant transformation. Physiol. Plant. 79:206–209.
22. Fitch, M. M. M., Manshardt, R. M., Gonsalves, D., Slightom, J. L. and Sanford, J. C. 1990. Stable transformation of papaya via microprojectile bombardment. Plant Cell Rep. 9:189–194.
23. Gonsalves, D. and Ishii, M. 1980. Purification and serology of papaya ringspot virus. Phytopathology 70:1028–1032.
24. An, G. 1986. Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells. Plant Physiol. 81:86–91.
25. Chee, P. P., Fober, K. A. and Slightom, J. L. 1989. Transformation of soybean (*Glycine max*) by infecting germinating seeds with *Agrobacterium tumefaciens*. Plant Physiol. 91:1212–1218.
26. Klein, T. M., Fromm, M., Weissinger, A., Tomes, D., Schaaf, S., Sletten, M. and Sanford, J. C. 1988. Transfer of foreign genes into intact maize cells using high velocity microprojectiles. Proc. Natl. Acad. Sci. USA 85:4305–4309.
27. McCabe, D. E., Swain, W. F., Martinell, B. J. and Christou, P. 1988. Stable transformation of soybean (*Glycine max*) by particle acceleration. Bio/Technology 6:923–926.
28. Slightom, J. L. 1991. Custom polymerase-chain-reaction engineering of a plant expression vector. Gene 100:251–255.
29. Slightom, J. L., Drong, R. F., Sieu, L. C. and Chee, P. P. 1991. Custom polymerase chain reaction engineering plant expression vectors and genes for plant expression 1–55. Plant Molec. Biol. Man. B16. S. B. Gelvin, R. A. Schilperoot and D. P. S. Verma (Eds.). Kluwer Academic Publishers, Dordrecht, The Netherlands.
30. Storey, W. B. 1953. Genetics of papaya. J. Hered. 44:70–78.
31. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.
32. Fitch, M. M. M. and Manshardt, R. M. 1990. Somatic embryogenesis and plant regeneration from immature zygotic embryos of papaya (*Carica papaya* L.). Plant Cell Rep. 9:320–324.
33. Fitch, M. M. M. 1992. High frequency somatic embryogenesis and plant regeneration from papaya hypocotyl callus. Plant Cell Tiss. Org. Cult. *In press.*
34. Lee, T. S. G. 1987. Micropropagation of sugarcane (*Saccharum* spp.). Plant Cell Tiss. Org. Cult. 10:47–55.
35. Jefferson, R. A. 1987. Assaying chimeric genes in plants. The GUS reporter gene fusion system. Plant Molec. Biol. Rep. 5:387–405.
36. Dellaporta, S. L., Wood, J. and Hicks, J. B. 1983. A plant DNA minipreparation: Version II. Plant Molec. Biol. Rep. 1:19–21.
37. Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A. and Allard, R. W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81:8014–8018.
38. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. 1988. Primer-directed amplification of DNA with a thermostable DNA polymerase. Science 239:487–491.
39. Maniatis, T., Fritsch E. F. and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, NY.
40. Maxodier, O., Cossart, P., Giraud, E. and Gasser, F. 1985. Completion of the nucleotide sequence of the central region of Tn*5* confirms the presence of three resistance genes. Nucl. Acids Res. 13:195–205.
41. Feinburg, A. P. and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analyt. Biochem. 132:6–13.
42. Napoli, C., Lemieux, C. and Jorgensen, R. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. The Plant Cell 2:279–289.
43. Sambrook, J., Fritsch, E. F. and Maniatis, T. 1991. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, NY.
44. Clark, M. F. and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay (ELISA) for the detection of plant viruses. J. Gen. Virol. 34:475–483.